High dose IL-2 skews a glucocorticoid-driven IL-17+IL-10+ memory CD4+ T cell response towards a single IL-10-producing phenotype

Short title: *Glucocorticoid-driven IL-10 is IL-2-dependent*

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Abstract

Glucocorticoids are known to increase production of the anti-inflammatory cytokine IL-10, and this action is associated with their clinical efficacy in asthmatics. However, glucocorticoids also enhance the synthesis of IL-17A by peripheral blood mononuclear cells, which in excess is associated with increased asthma severity and glucocorticoid-refractory disease. Here we show that the glucocorticoid dexamethasone significantly increased IL-10 production by human memory $CD4^+$ T cells from healthy donors, as assessed by intracellular cytokine staining. In addition, dexamethasone increased production of IL-17A, IL-17F and IL-22, with the most striking enhancement in cells co-producing Th17-associated cytokines together with IL-10. Of note, an increase in $IFN\gamma^+IL\text{-}10^+$ cells was also observed despite overall downregulation of IFNγ production. These dexamethasone-driven $IL\text{-}10^+$ and predominantly the $IL\text{-}17^+IL\text{-}10^+$ double-producing cells were markedly refractory to the inhibitory effect of dexamethasone on proliferation and IL-2Rα expression, which facilitated their preferential IL-2-dependent expansion. Whilst lower concentrations of exogenous IL-2 promoted $IL\text{-}10^+$ cells co-producing pro-inflammatory cytokines, higher IL-2 doses, both alone and in combination with dexamethasone increased the proportion of single $IL\text{-}10^+$ T cells. Thus, glucocorticoid-induced IL-10 is only accompanied by an increase of IL-17 in a low IL-2 setting, which is nevertheless likely to be protective owing to the induction of regulatory $IL\text{-}17^+IL\text{-}10^+$ co-producing cells. These findings open new avenues of investigation with respect to the role of IL-2 in glucocorticoid responsiveness that have potential implications for optimising the benefit-risk ratio of glucocorticoids in the clinic.

Introduction

Glucocorticoids are a class of lipophilic steroid hormones that are synthesised endogenously by the adrenal cortex. They can bind to the glucocorticoid receptor (GR), which is expressed by most nucleated cells, and trigger a broad range of effects via transactivation and transrepression, in addition to other GR-independent actions. Their actions are pleiotropic, affecting various physiological processes including development, metabolism and inflammation, and as such, synthetic glucocorticoids have been used in the clinic since 1948 (1). Glucocorticoids remain the most important anti-inflammatory pharmacotherapy in modern medicine, despite their untoward side-effects. Their anti-inflammatory properties result from their transrepression of pro-inflammatory genes such as interleukin (IL)-1β and IL-4, transactivation of anti-inflammatory genes and upregulation of the frequency and activity of regulatory T cells (Tregs) (2). *In vivo* glucocorticoids have been shown to increase serum levels of the anti-inflammatory cytokine IL-10 (3) as well as the synthesis of IL-10 by cells locally in the airways (4). Furthermore, the synthetic glucocorticoid dexamethasone enhances the concentration of IL-10 in cultures of peripheral blood mononuclear cells (PBMCs), $CD4^+$ and $CD8^+$ T cells isolated from healthy humans *in vitro* (5-8).

The importance of glucocorticoid-induced IL-10 is highlighted by studies in patients with severe steroid-resistant (SR) asthma, who represent a profound clinical challenge for disease management. SR asthma patients have a defect in the dexamethasone-driven IL-10 response (6, 9, 10) and heightened levels of IL-17A; indeed levels of IL-17A inversely correlate with lung function (11) and are significantly elevated in the peripheral blood (6, 7, 12), sputum (13), serum (14, 15) and bronchial alveolar lining fluid (16, 17) of patients with severe asthma, with the greatest levels observed in patients with SR disease (7). Levels of IL-17A are also elevated in mouse models of airway hyperresponsiveness where Th17 cells drive pathology (18, 19).

Th17 cells are critical for protecting against mucosal and fungal infections, however they have also been implicated in various immune-mediated diseases (20). More specifically, cells that differentiate in the presence of IL-23 and TGF-β3 to co-express Th1- and Th17-associated molecules have been shown to drive experimental autoimmune encephalomyelitis in mice (21, 22). Ramesh *et al*. showed human peripheral blood $CD4^+$ T cells cultured with IL-23 produced IL-17A, IL-17F, IL-22 and IFN-γ but not IL-10 (23). However distinct Th17 phenotypes exist, for example Zielinski *et al*. observed *Candida albicans*-specific human $CD4^+$ T cells producing IL-17A and IFN-γ but not IL-10, and *Staphylococcus aureus*-specific cells that in contrast could produce IL-10 (24). Functionally, Th17 cells that co-produce IL-10 have been shown to restrain Th17-cell mediated pathology (21, 25-27)

In order to better understand these seemingly opposing effects of glucocorticoids on the induction of IL-10 and IL-17A, this study sought to characterise the cellular source of glucocorticoid-driven IL-10 and investigate its co-expression with other cytokines, based on emerging data of functionally distinct Th17 subsets. We set out to dissect the mechanism for induction of IL-10 versus IL-17 in T cells, which are critical targets in inflammatory tissues, such as the airways in asthma. This study identifies that glucocorticoids enhance the proportion of memory $CD4^+$ T cells that co-produce IL-17^+IL-10^+, which is reflective of a less inflammatory, protective Th17 phenotype. Notably, addition of higher dose exogenous IL-2 skews this response towards a single IL-10-producing phenotype, which has potential implications for generating more targeted therapeutics.

Materials and Methods

Cell isolation and culture

Ethical approval was granted by London Bridge National Research Ethics Service Ethics Committee (REC 09/H0804/77 and REC 14/LO/16990) and HTA license number 12650 at the Francis Crick Institute, and full written informed consent obtained from all donors. Peripheral venous blood was collected from non-asthmatic male and female healthy adults into syringes containing sodium citrate (10:1). Peripheral blood mononuclear cells (PBMCs) isolated using Lymphoprep as previously described (8). $CD45RO^+$ memory $CD4^+$ T cells were purified by negative selection (Miltenyi Biotec; Bisley, UK; >97% $CD4^+CD45RO^+$). Cells were re-suspended at a concentration of $1x10^6$ cells/ml in RPMI 1640 growth medium containing 10% FBS, 2 mM L-glutamine and 50 μg/ml gentamycin.

$0.5x10^6$ cells were stimulated with 1 μg/ml plate-bound anti-CD3 (clone OKT-3) and 50 IU/ml rhIL-2 (Eurocetus, Harefield, UK) for 5 days unless otherwise stated in a 48-well plate at 37°C (5% CO_2). On day 3, half of the supernatant was removed and replaced with full media supplemented with rhIL-2. 100 nM dexamethasone (Sigma-Aldrich; Gillingham, UK) or 0.01% ethanol vehicle control was added to the cell cultures where indicated. 10 μg/ml anti-IL-2 (aIL-2; clone 5334; R&D Systems) was added on days 0 and 3 where indicated.

Cell proliferation

Prior to culture cells were labeled with 1 μM CellTrace Violet as per the manufacturer's instructions (ThermoFisher). Cell proliferation was assessed by loss of fluorescence intensity on an NxT Attune (ThermoFisher) or Fortessa (BD Biosciences).

Surface Staining

Cells were harvested and washed in 2% FCS-PBS prior to performing viability (Zombie Violet/Aqua viability dye (Life Technologies)) and surface staining on ice in the dark for 30 minutes. The following antibodies were used for cell surface phenotyping: CD4-PerCPCy5.5, CD45RA-APCCy7, CD45RO-PECy7 (clones OKT4, HI100 and UCHL1 respectively; Biolegend), CD3-V500 CD25-APCH7 (clones SP34-2 and M-A251 respectively; BD Biosciences), CD122-FITC (TU27; eBiosciences). Cells were washed a further two times in 2% FCS-PBS and then fluorescence assessed using an NxT Attune or Fortessa.

Intracellular staining

Human cells were stimulated with 5 ng/ml PMA (phorbol-12-myristate-13-acetate) and 500 ng/ml Iononomycin (Sigma Aldrich) for 4 hours at 37°C, with 2 μM Monensin added the final 3 hours. Cells were surface stained on ice with the relevant markers and aqua zombie viability dye (BioLegend) for 30 minutes before fixing and permeabilising using BD FixPerm kit as per the manufacturer's instructions. The following antibodies were used to stain intracellular molecules: IL-22-PECy7 (22URTI), IL-22-PE (22URTI) IL-4-PECy7 (8D4-8), IL-17A-APC (eBio64Dec17), IL-17F-aF488 (Poly5166) and IL-2-PerCPCy5.5 (MQ1-17H12), all from eBiosciences; IFN-γ-FITC (4S.B3) and IL-10-PE (JEs3-9D7) from BD Biosciences. Cells were washed a further two times in 2% FCS-PBS and then fluorescence assessed using an NxT Attune or Fortessa.

Phospho-STAT5 flow cytometry

Cells were rested in full media for 2 hours and then stimulated with 5ng/ml rhIL-2 for 30 minutes. Samples were then surface stained and fixed prior to re-suspending BD Phosflow Perm Buffer III. Samples were incubated in Perm Buffer III on ice for 30 minutes and then washed twice in Perm Buffer III before staining with the phospho-STAT5 antibody (Alexa

Fluor 488; 47/Stat5 (pY694); BD Biosciences) for a further 30 minutes on ice. Cells were washed a further two times in 2% FCS-PBS and then fluorescence assessed using an NxT Attune or Fortessa.

Data analysis

Flow cytometry data were analysed using FlowJo (Treestar Inc.; version 10). Singlet events (based on both forward and side scatter area and height comparison) and viable (Zombie Aqua negative) cells were selected prior to analysis. Cumulative data analysis was performed in Graphpad Prism version 7.00 for Mac OS X (Graphpad Software Inc., San Diego, USA). After assessing for a Gaussian distribution, an appropriate statistical test was performed as described in the figure legends at the 95% confidence level. Data are shown as mean +/- standard error of the mean (SEM).

Results

Dexamethasone increases production of IL-10 by human memory CD4$^+$ T cells

We have previously shown that the glucocorticoid dexamethasone increases the concentration of IL-10 in the supernatants of anti-CD3/IL-2-stimulated CD8-depleted PBMC, CD4$^+$ T cell and CD8$^+$ T cell cultures (5-8). To characterise which T cell subpopulation showed the greatest induction of IL-10 expression by dexamethasone, PBMCs were stimulated with anti-CD3 and IL-2 T cell stimulation in the absence or presence of 10^{-7}M dexamethasone. Dexamethasone increased the frequency of IL-10-producing cells to the greatest extent in the CD45RO$^+$ memory compartment of CD4$^+$ PBMCs following 5 days stimulation as assessed by intracellular cytokine staining (Figure 1A and B; Supplementary Figure 1). Consistent with this, in pure memory CD4$^+$ T cell cultures a greater frequency of cells expressed IL-10 and this was dose-dependently upregulated by dexamethasone (Figure 1C).

Dexamethasone enhances production of IL-10 and IL-17A, but not IFNγ or IL-4

The kinetics of the dexamethasone-driven IL-10 response was next investigated directly in memory CD4$^+$ T cells stimulated over a 6-day culture period (Figure 2). In the absence of dexamethasone, the frequency of IL-10-producing cells reduced over time. In contrast, addition of 10^{-7}M dexamethasone significantly increased the frequency of IL-10$^+$ cells by day 5, although not at earlier time points. The proportion of IL-17A$^+$ cells gradually increased with time and dexamethasone significantly, albeit more modestly, further enhanced the frequency of IL-17A$^+$ T cells at days 5 and 6 of culture (Figure 2A). In contrast, expression of IFNγ, IL-4 and IL-2 was reduced or unaltered by dexamethasone throughout the culture (Figure 2A and B). These findings are in keeping with our previous findings (6, 7, 12) and further demonstrate that memory CD4$^+$ T cells are the cellular source of both IL-10 and IL-17 following dexamethasone treatment.

Dexamethasone induces co-production of IL-10 with multiple pro-inflammatory cytokines

IL-10$^+$CD4$^+$ T cells can co-produce multiple other pro-inflammatory cytokines in a heterogeneous manner (28), including IL-17A (21, 24-27). We therefore investigated the effect of dexamethasone on other Th17-associated cytokines, as well as the co-production of cytokines with IL-10 by memory CD4$^+$ T cells. Dexamethasone enriched the proportion of memory CD4$^+$ T cells producing the Th17 family members IL-17F and IL-22 (Figure 3A and B; Supplementary Figure 2A). The average fold change in IL-17A, IL-17F and IL-22 expression was 1.9-, 4.3- and 1.4-fold respectively, and that of IL-10$^+$ cells was 4.9-fold, relative to the vehicle control (Supplementary Figure 2B). Notably greater fold increases were consistently observed in cells co-producing IL-10 together with Th17-associated cytokines, as compared to their IL-10$^-$ counterparts (Supplementary Figure 2B). Dexamethasone significantly reduced the frequency of IFNγ$^+$ cells that did not co-produce IL-10 but induced a 7.5-fold increase in the proportion of IFNγ$^+$IL-10$^+$ memory CD4$^+$ T cells, resulting in no change in the overall proportion of IFNγ$^+$ memory CD4$^+$ T cells. A similar trend was observed for production of IL-4 and IL-2 by dexamethasone-treated memory CD4$^+$ T cells, with an enhanced proportion of cells co-producing IL-10 in contrast to a reduced frequency of IL-10$^-$ cells.

Since it has been reported that inflammatory Th17 cells may co-produce inflammatory cytokines such as IFNγ (21-23), the dexamethasone-stimulated cells analysed for IL-10 and IL-17A production were further investigated for co-production of IL-17F (yellow); IL-22 (red); IFNγ (blue); IL-4 (green); and IL-2 (orange) (Figure 3C). As predicted, IL-17F (yellow) was predominantly produced by IL-17A$^+$ cells, irrespective of whether they were IL-10$^-$ or IL-10$^+$. Although IL-22$^+$ cells (red) were present in both the IL-10$^+$ and IL-10$^-$ populations, most did

not co-produce IL-17A. The majority of IFNγ$^+$ (blue) or IL-4$^+$ (green) cells did not co-produce IL-17A but many co-expressed IL-10$^+$. IL-2$^+$ (orange) cells were predominantly negative for IL-17A and IL-10 (Figure 3C). Therefore, dexamethasone dominantly drives the production of IL-10 across all memory CD4$^+$ T cell subsets, excluding those producing IL-2. Notably, the dexamethasone-stimulated IL-17A-producing cells were predominantly negative for IFNγ, IL-4 and IL-2, although a large proportion co-produced IL-10, which is indicative of a non-pathogenic Th17 phenotype (25-27, 29).

IL-10$^+$ cells are refractory to the suppressive effects of dexamethasone on cell proliferation

To investigate the mechanisms driving the enrichment of IL-10$^+$ cells within dexamethasone-treated memory CD4$^+$ T cell cultures, cytokine expression was assessed in relation to cell proliferation. Memory CD4$^+$ T cells were labelled with the fluorescent dye CellTrace Violet prior to stimulation in the presence or absence of dexamethasone. IL-10 and IL-17A were produced predominantly by cells that had undergone multiple rounds of cell division (Figure 4A) and were enhanced in the presence of dexamethasone (Figure 4A and B). In contrast IFNγ and IL-4 production was seen in both dividing and non-dividing cells, but their frequency was not altered by dexamethasone. IL-2 was produced primarily by the undivided population and was decreased by dexamethasone (Figure 4 and B).

Glucocorticoids have been repeatedly shown to dampen T cell proliferation (30), which we now show for total memory CD4$^+$ T cells (“All cells” Figure 4C and D). However, cells producing IL-10 and/or IL-17A were refractory to this suppressive effect of dexamethasone on cell proliferation (Figure 4C and D). This contrasts with the proliferation of IFNγ$^+$, IL-4$^+$ and IL-2$^+$ cells which was significantly reduced in dexamethasone-treated cultures (Figure 4C and

D). Notably the proliferation of all IL-10$^+$ memory CD4+ T cells, regardless of whether they co-produced IFNγ, IL-4 or IL-2, was not suppressed by dexamethasone, in marked contrast to their IL-10$^-$ counterparts (Figure 4C and D). These data show that IL-17A$^+$ and IL-10$^+$ cells are markedly refractory to the anti-proliferative effects of dexamethasone.

IL-10$^+$ memory CD4$^+$ T cells are resistant to down-regulation of the IL-2 receptor by dexamethasone

IL-2 plays a central role in supporting the proliferation of T cells (31, 32) and its production is known to be inhibited by dexamethasone (33). Therefore, the contribution of this pathway to the observed differential effect of dexamethasone on IL-10$^+$ versus IL-10$^-$ cells was next investigated. Cell surface expression of IL-2 receptor components CD25 (high affinity IL-2Rα) and CD122 (IL-2Rβ) increased upon stimulation over time and this increase was inhibited by dexamethasone (Figure 5A and B). Dexamethasone-treated memory CD4$^+$ T cells also showed lower amounts of downstream STAT5 phosphorylation as compared to vehicle control. The cells that had upregulated CD25 proceeded into cell division regardless of the action of dexamethasone (Figure 5C). Moreover, the percentage of CD25$^+$ cells was higher on the IL-10$^+$ cells as compared to IL-10$^-$ cells, suggesting that IL-10$^+$ cells are selectively resistant to modulation of CD25 by dexamethasone (Figure 5D). This provides a possible explanation for our findings that IL-10$^+$ memory CD4$^+$ T cells continue to proliferate in the presence of dexamethasone.

High dose IL-2 maintains dexamethasone-induced IL-10 whilst inhibiting IL-17A in memory CD4$^+$ T cells

In the experiments described above 50 IU/ml rhIL-2 was routinely added to all cultures. To further investigate the resistance of IL-10$^+$ cells to downregulation of the IL-2 receptor CD25

and continued proliferation in the presence of dexamethasone, we investigated the contribution of IL-2 to this process. Dexamethasone induction of IL-10 required the addition of rhIL-2, with no dexamethasone-mediated enhancement of IL-10 observed in the presence of anti-IL-2 neutralising monoclonal antibody (mAb) due to its suppressive effect on proliferation (Figure 6A and B). In the absence of dexamethasone, addition of anti-IL-2 mAb to the cultures led to a consistent decrease in the frequency of cells producing IL-10 and all effector cytokines measured, with the exception of IL-2 which was shown to be increased as compared to the vehicle control without IL-2 intervention (Figure 6A; data not shown for IFNγ and IL-4). Conversely, increasing concentrations of rhIL-2 in the absence of dexamethasone dose-dependently increased the frequency of IL-10-producing cells, whilst reducing that of IL-2-producing cells. Nevertheless, at all concentrations of IL-2 studied, 50 – 1000 IU/ml, the addition of dexamethasone further enhanced the frequency of memory $CD4^+$ T cells producing IL-10 without affecting levels of IL-2 (Figure 6A and B).

Enhancement of the frequency of IL-$17A^+$ T cells by dexamethasone was also IL-2-dependent, as it was not observed either in the presence of anti-IL-2 or without addition of exogenous IL-2 to the cultures (Figure 6A). In contrast to IL-10, however, the capacity of dexamethasone to increase IL-17A production was only seen at the lower concentration of IL-2 studied and was inhibited at the higher concentrations of IL-2 (500-1,000 U/ml). Therefore higher-dose rhIL-2 importantly maintained the elevated IL-10 response to dexamethasone, whilst constraining the inductive effect of dexamethasone on IL-17A. As observed for IL-17A, low-dose, but not higher concentrations of rhIL-2 were required for dexamethasone to enhance the frequency of memory $CD4^+$ T cells co-producing IL-10 and IL-17A (Figure 6A). Furthermore, when higher doses of rhIL-2 was added to the cultures, dexamethasone increased IL-10 production by both dividing and un-dividing cells (Figure 6B and C). Thus, our data suggest that IL-2 can

independently upregulate IL-10 in the absence of dexamethasone, and that dexamethasone further increases IL-10 over-and-above the effect of IL-2. Notably, the combination of glucocorticoids and a higher dose of IL-2 promoted an IL-10^{+} memory $CD4^{+}$ T cell population with minimal co-production of pro-inflammatory cytokines.

Discussion

Glucocorticoids are the cornerstone of treatment for patients with asthma and many other inflammatory conditions, and their clinical efficacy is linked in part to their capacity to induce synthesis of the anti-inflammatory cytokine IL-10 (9). To further expand our understanding of glucocorticoid action, we used cells from healthy individuals who respond to dexamethasone by increases in IL-10 and IL-17A but are devoid of any confounding asthma-associated defects (7). We show that a major source of glucocorticoid-induced IL-10 synthesis is $CD4^+$ memory T cells, which are abundant in tissues such as the lung (34). Glucocorticoids enhanced the frequency of IL-10-expressing T cells, an effect that was particularly marked in T cells co-expressing IL-10 along with a range of effector, including Th17-associated cytokines. The glucocorticoid dexamethasone concurrently increased the frequency of cells producing IL-17A, resulting largely from enrichment in the frequency of $IL\text{-}17A^+IL\text{-}10^+$ cells. Notably, cells producing IL-10 and/or IL-17A, including $IFN\gamma^+IL\text{-}10^+$ but not $IFN\gamma^+IL\text{-}10^-$ cells, were refractory to the anti-proliferative effects of dexamethasone. This glucocorticoid-induced IL-10 production was dependent on IL-2, which itself could independently upregulate IL-10. Higher doses of recombinant IL-2, whilst sustaining IL-10 production, inhibited the dexamethasone-induced $IL\text{-}17A^+$ response.

IL-10-producing Th17 cells are indicative of the non-pathogenic Th17 cell population that has been shown to have a critical role in restraining Th17 cell-mediated inflammatory and autoimmune diseases (25-27). This contrasts with pro-inflammatory $IFN\gamma^+$Th17 cells that have been implicated in both murine (21, 22) and human (23) pathologies. In addition to inducing $IL\text{-}10^+IL\text{-}17^+$ T cells, we show here that dexamethasone also induced $IL\text{-}10^+IL\text{-}22^+$ cells. Since Th17-associated cytokines play a central role in defence against extracellular infections at mucosal sites, we propose that in tissues such as the lung, the dual capacity of glucocorticoids

to both maintain at least certain anti-microbial defence mechanisms via IL-17A and IL-22, as well as dampen inflammation via IL-10, is highly desirable. It would therefore be interesting to study the co-production of these cytokines over the course of a range of diseases in humans; in mice IL-22 has been show to drive the initiation of airway inflammation but act as a negative regulator in established disease (35). We also observed that dexamethasone selectively enriched a population of IFNγ$^+$IL-10$^+$ cells which we hypothesise would dampen excessive airway inflammation. Indeed, our earlier studies in steroid-refractory patients indicate that both IL-17A and IFNγ, but not IL-10, synthesis are significantly elevated compared to steroid-sensitive patients (12).

The capacity of dexamethasone to enhance the frequency of cells co-producing IL-10 with Th17-associated cytokines reflected preferential outgrowth of this population in culture and was dependent on the presence of the growth factor IL-2. Only the lower concentration of IL-2 studied promoted the expansion of IL-17A$^+$ or IL-17A$^+$IL-10$^+$ cells. Low-dose IL-2 is required for T cell proliferation and survival (31, 32), but increased IL-2 signalling is proposed to impair IL-17A responses as pSTAT5 is reported to compete with pSTAT3 for binding to the *il17a* gene locus (36, 37). Therefore, the fact that dexamethasone down regulated, but importantly did not eliminate expression of IL-2, IL-2Rα1, IL-2Rβ and pSTAT5 may favour the outgrowth of Th17 cells. However, we show that higher doses of IL-2 overcame this to inhibit dexamethasone-induced IL-17A although sustaining IL-10-producing cells. Hence our findings provide a novel approach to promoting non-pathogenic Th17 cells which co-produce IL-10. Of note, as well as driving the outgrowth of IL-10$^+$ cells, the higher concentrations of rhIL-2 studied promoted the expansion of a population of cells that were predominantly negative for all cytokines assessed (data not shown), and may reflect a central memory T cell population (38).

The mechanisms by which dexamethasone increases the synthesis of IL-10 and IL-17A are likely to be distinct given the pleiotropic nature of glucocorticoid actions. The relatively slow nature of both responses suggests that they are unlikely to be due to a dexamethasone-glucocorticoid receptor complex binding directly to target DNA, although glucocorticoid response elements (GREs; consensus motif from Hocomoco, Jaspar, (39)) are located both up- and down-stream of the *il10* promoter. GREs are also found within the vicinity of the *il2*, *il2ra*, *il2rb* and *stat5* loci, suggesting that dexamethasone may directly influence the transcription of these genes. Increased production of Th17-associated cytokines may also be in part due to reduced Th1 and Th2 responses, both of which have been shown to reduce IL-17A production (40, 41). Using the same experimental system we previously reported that addition of IL-4 to the culture attenuated the IL-17A response (12). Conversely, neutralising IL-4, but not IFNγ, in culture increased levels of IL-17A in the control condition to levels comparable of dexamethasone-treated cells, which was unaffected by IL-4 blockade (data not shown).

Minimal suppression by glucocorticoids of Th17 cell proliferation, as shown here, has also been observed in murine *in vitro* and *in vivo* systems when compared to Th1 and Th2 cells (18, 42), and human pro-inflammatory Th17 cells have been shown to be refractory to dexamethasone's suppressive effects on proliferation (23). One potential mechanism for this identified in this study is that cells co-producing IL-17A and IL-10 expressed elevated levels of CD25 which were refractory to inhibition by dexamethasone, in contrast to cells not making IL-10, enabling their preferential expansion. In line with this observation, it has been shown that only a subset of $CD4^+$ T cells expressing elevated levels of CD25 continue to proliferate in the presence of dexamethasone, although cytokine expression was not assessed in that study (43). Our novel findings show that $IL\text{-}10^+$ cells, irrespective of whether they co-produce pro-

inflammatory cytokines, can maintain IL-2 signaling and proliferate in the presence of glucocorticoids.

Our previous studies identify glucocorticoid-induced IL-17A as a feature of steroid-resistant severe asthma (12), but here we address the underlying mechanisms and single-cell phenotyping. Our data suggest that aberrant glucocorticoid-driven IL-17A in steroid-resistant asthma may be a result of the immune environment in these patients, and in particular low but not absent levels of IL-2. Indeed, daily glucocorticoid therapy might dampen IL-2 receptor expression and signalling to favour Th17 differentiation and/or expansion, particularly in the setting of weak T cell stimulation, such as during sub-clinical airways infection with microbes such as Haemophilus, which is associated with severe asthma (44). To this end, severe asthmatics were found to have elevated soluble CD25 in circulation and increased expression by $CD4^+$ T cells, which inversely correlated with airway obstruction (45, 46).

Although not studied in asthma, low-dose IL-2 therapy has been found beneficial in patients with diseases including graft-versus-host disease (GVHD), many of whom were receiving glucocorticoids (47), and hepatitis C virus-induced vasculitis (48). Such beneficial effects may be due to IL-2 dampening Th17 cells, which is hypothesised to be beneficial in GVHD (49). However much of the research emphasis has been in describing the expansion of $Foxp3^+$ Tregs following IL-2 therapy (47, 50, 51). Co-expression of Foxp3 and IL-10 was not assessed in this study but dexamethasone-driven IL-10 was not accompanied by a change in Foxp3 mRNA or protein expression (data not shown), suggesting that the cells in this study were not conventional Tregs. This is in line with both our own and independent reports (52-54) that observe very low levels of co-expression of Foxp3 and IL-10 protein in human CD4+ T cells. Our findings showing that IL-2 increases IL-10-producing memory $CD4^+$ T cells may also

provide an explanation for the defective IL-10 production, but normal Foxp3 gene expression, in $CD4^+$ T cells from a patient with CD25 deficiency manifesting as immune dysregulation, polyendocrinopathy, enteropathy and X-linked-like syndrome (55).

We have previously shown that vitamin D can restore dexamethasone-induced IL-10 production in severe asthma (10, 12), but whether combining IL-2 therapy with glucocorticoids to further enhance IL-10 production and suppress IL-17A production is a pharmacotherapeutic option in severe asthma needs further consideration. Firstly, IL-17A, and other Th17-associated cytokines, have both beneficial and harmful actions. Secondly, one of the main limitations to IL-2 therapies is the profound differences in immunologic effects of different IL-2 doses, with pro-inflammatory actions observed at the highest doses. Interestingly Sockolosky and colleagues (56) recently published on the generation of orthogonal IL-2 cytokine-receptor complexes that selectively target cell populations for adoptive cell therapy. Although the study did not measure its effects on levels of IL-10 and Th17-associated cytokines, they showed that IL-2, but not the orthogonal IL-2 complex increased the serum levels of several pro-inflammatory cytokines in mice which is associated with serious side effects. It would therefore be interesting to investigate whether this may be a mechanism to therapeutically skew responses towards an IL-10-single-producing response, and any impact of common therapeutic agents such as glucocorticoids upon this process.

In summary, we show that dexamethasone promotes the preferential proliferation of $IL\text{-}10^+$ and $IL\text{-}17A^+$ memory $CD4^+$ T cells, specifically enriching for populations of cells co-producing IL-10 and multiple pro-inflammatory cytokines. Increasing the concentration of rhIL-2 in the presence of dexamethasone maintained elevated levels of IL-10 whilst reducing the IL-17A

response, which may provide new avenues for the development of improved clinical therapies for asthma.

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Conflicts of interest

The authors declare no competing financial interests.

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Figure legends

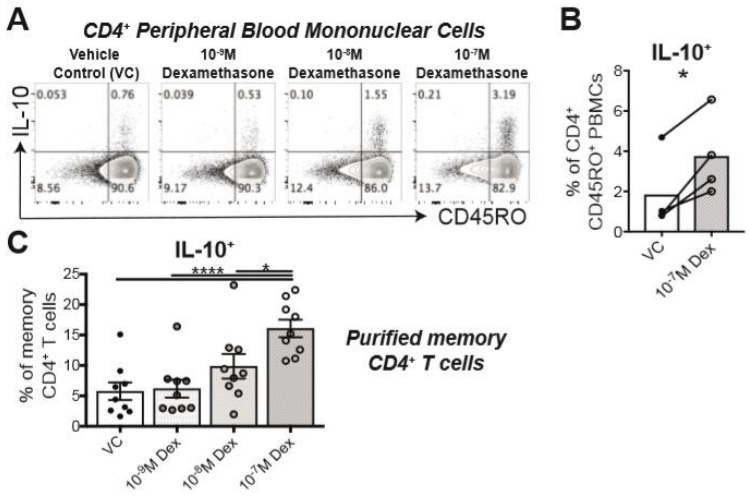


Figure 1: Glucocorticoids enhance production of IL-10 in memory CD4$^+$ T cells.

PBMCs (**A** and **B**) or memory CD4$^+$ T cells (**C**) from healthy donors were stimulated with 10^{-9}M-10^{-7}M dexamethasone or control for 5 days. Cells were then stimulated for 4 hours with PMA and Ionomycin prior to staining for surface markers and intracellular IL-10. For PBMC cultures, shown are representative plots (**A**) and cumulative data (**B**) gating of CD4$^+$CD45RO$^+$ cells (n=4); data assessed data assessed by a paired t-test. **C**, the percentage of IL-10$^+$ cells in memory CD4$^+$ T cell cultures (n=9); data assessed by repeated measures one-way ANOVA with Tukey's multiple comparisons test. * $p \leq 0.05$, **** $p \leq 0.0001$.

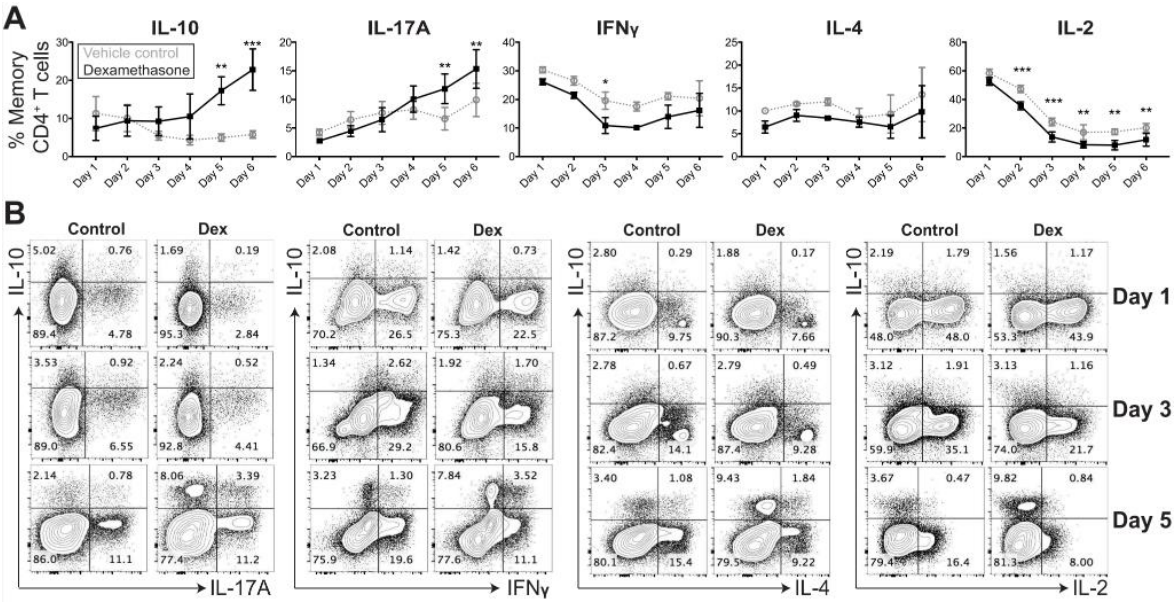


Figure 2: Glucocorticoids increase expression of IL-10 and IL-17A, but not IFNγ, IL-4 or IL-2, in memory CD4$^+$ T cell cultures

Memory CD4$^+$ T cells were stimulated in the presence of vehicle control (grey) or 10^{-7}M dexamethasone (black). On the indicated day, cells were stimulated for 4 hours with PMA and Ionomycin to assess intracellular cytokine expression. Shown are cumulative data (**A**; n=4 except IL-4, n=2) and representative plots (**B**). Data assessed by a two-way ANOVA with Sidak's multiple comparisons test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

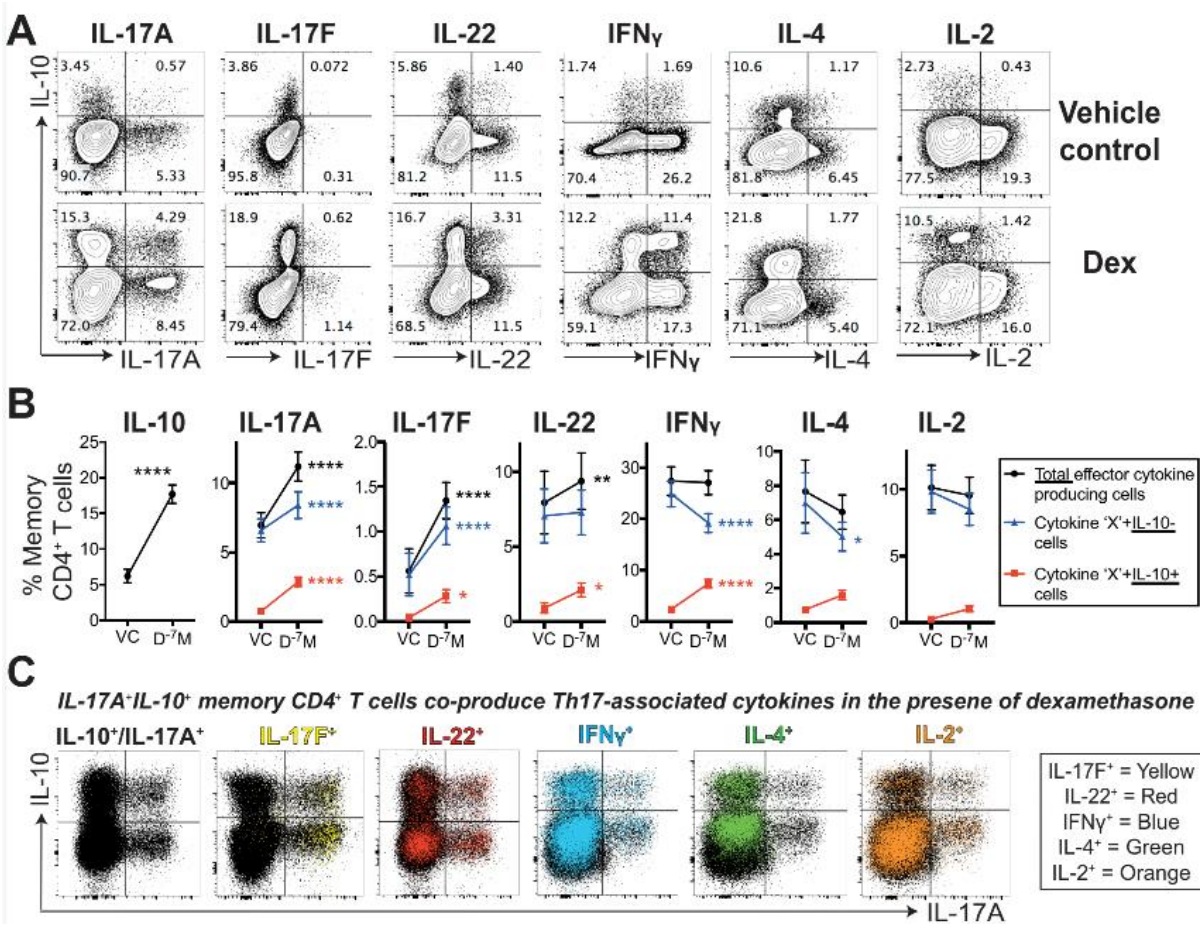


Figure 3: Co-production of IL-10 and pro-inflammatory cytokines is induced by glucocorticoids in memory CD4$^+$ T cells

Memory CD4$^+$ T cells were stimulated in the presence of vehicle control or 10^{-7}M dexamethasone. On day 5 cells were stimulated for 4 hours with PMA and Ionomycin prior to performing intracellular cytokine staining. **A**, representative contour plots. **B**, the total (black) percentage of cells producing the indicated cytokine alongside those co-expressing IL-10 (red) or the IL-10$^-$ counterparts (blue); data assessed by two-way ANOVA with Sidak's multiple comparison's test (IL-10 n=32; IL-17A n=29; IL-17F n=7; IL-22 n=6; IFNγ n=25; IL-4 n=6; IL-2 n=19). **C**, representative overlay dot plots identifying which cells within the IL-10/IL-17A plots (black; far left) co-produced the indicated cytokine in colour (IL-17F in yellow, IL-22 in red, IFNγ in blue and IL-4 in green). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

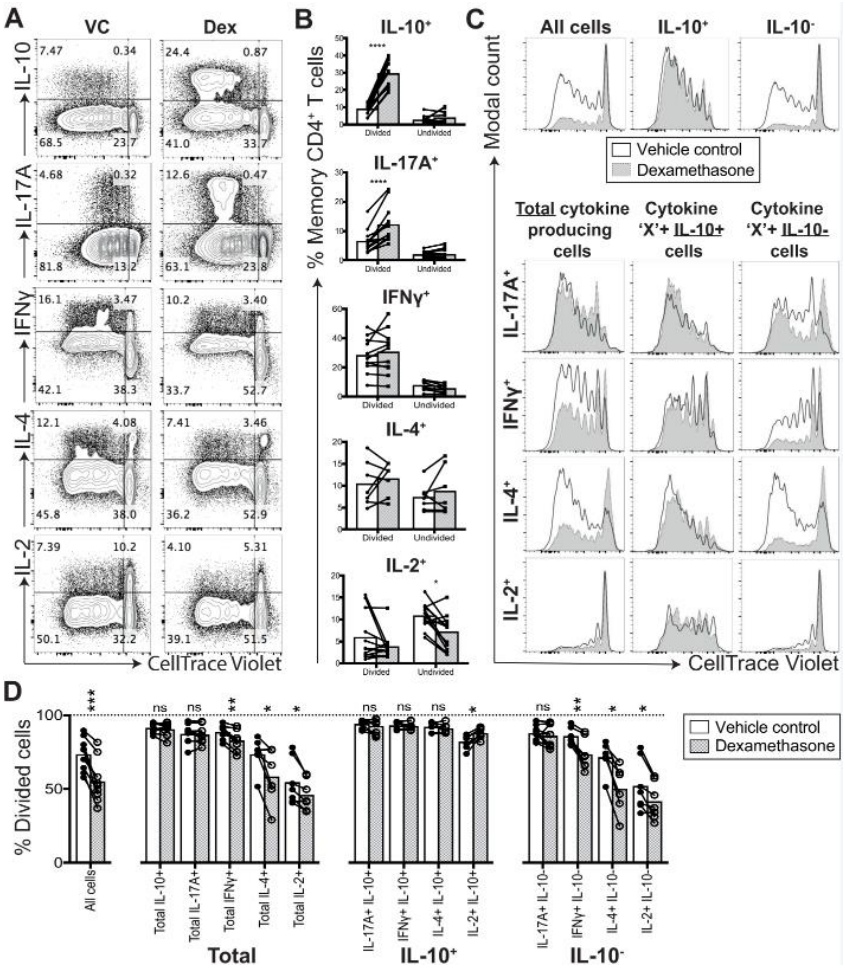


Figure 4: IL-17A$^+$ cells and cells co-producing IL-10 are refractory to the suppressive effects of glucocorticoids on cell proliferation

Memory CD4$^+$ T cells were labelled with CellTrace Violet on day 0 and then stimulated in the presence of vehicle control (white) or in the presence of 10^{-7}M dexamethasone (grey). On day 5 cells were stimulated for 4 hours with PMA and Ionomycin prior to performing intracellular cytokine staining. **A**, representative plots showing expression of cytokines relative to cell proliferation. **B**, cumulative data of the percentage of cells expressing the indicated cytokine within the divided or undivided cell population; data assessed by a two-way ANOVA with Sidak's multiple comparisons test. **C**, representative histograms showing the proliferation of cells when gating on the indicated cytokine-expressing populations, alongside cumulative data (**D**); data assessed by a paired t-test. IL-17A and IL-10 n=9; IFNγ/IL-2 n=7; IL-4 n=6; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

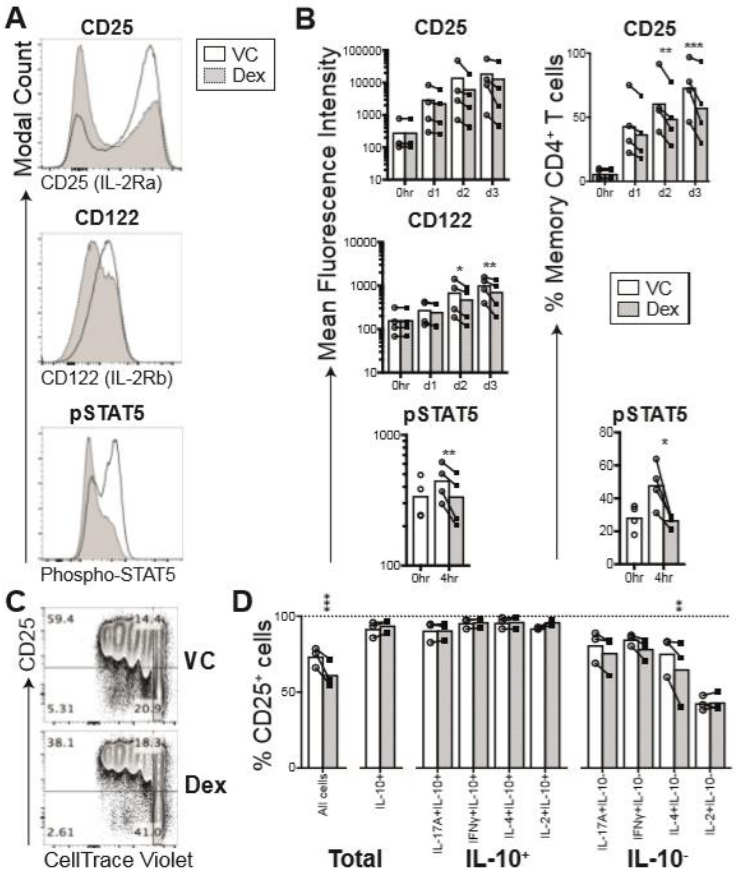


Figure 5: Glucocorticoids dampen IL-2 signalling in memory CD4$^+$ T cells

Memory CD4$^+$ T cells were stimulated in the presence of vehicle control (whit) or 10^{-7}M dexamethasone (grey). **A** and **B**, after the indicated length of time, cells were surface stained for CD25 (IL-2Ra; top) and CD122 (IL-2Rb; middle); for phospho-STAT5 staining, cells were rested for 2 hours, stimulated with 5ng/ml rhIL-2 for 30 minutes and then phospho-STAT5 expression assessed. Shown are representative overlay histograms (**A**) alongside cumulative data for the mean fluorescence intensity and the percentage of positive cells (**B**; n=4). **C**, representative plots showing expression of CD25 by cells relative to their proliferation status (Cell Trace violet) on day 5. **D**, on day 5 cells were stimulated for 4 hours with PMA and Ionomycin prior to surface CD25 and intracellular cytokine staining (n=3); shown is the % of CD25$^+$ cells when gating on all cells (left) or the indicated cytokine-expressing populations (n=3). Data assessed by a two-way ANOVA with Sidak's multiple comparisons test; ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

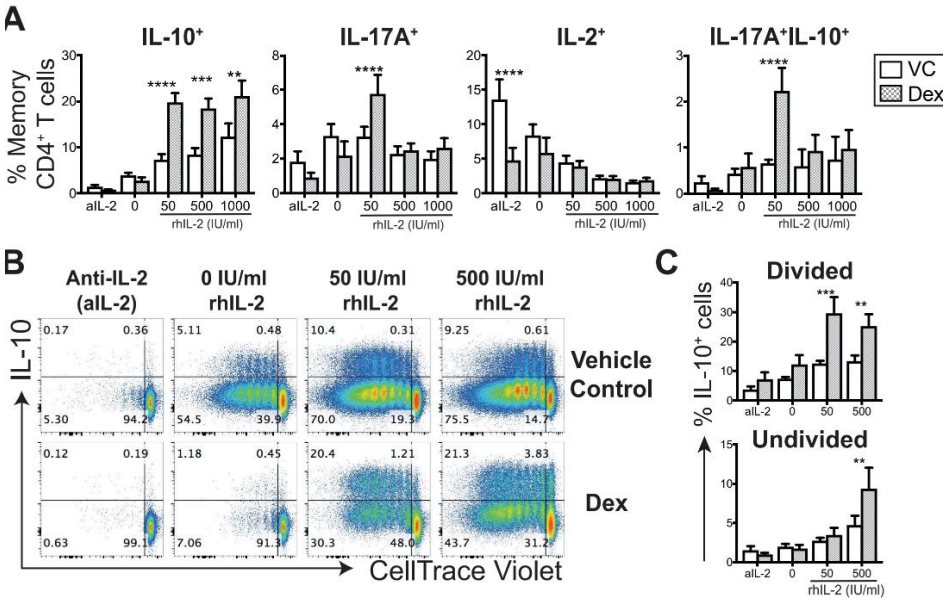
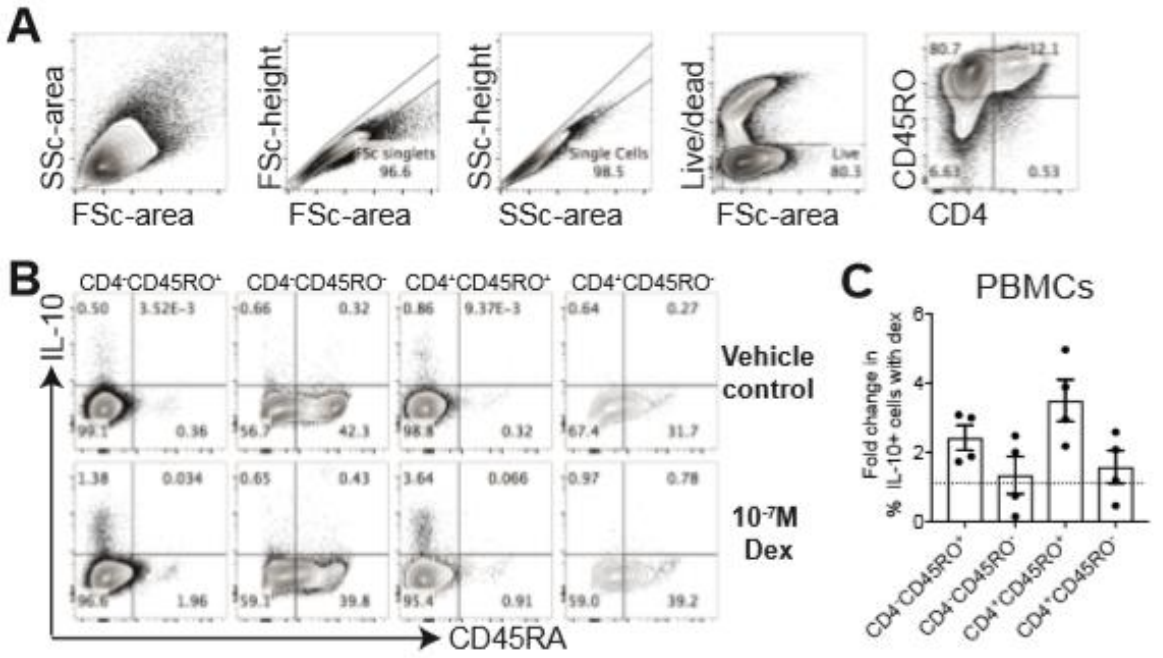
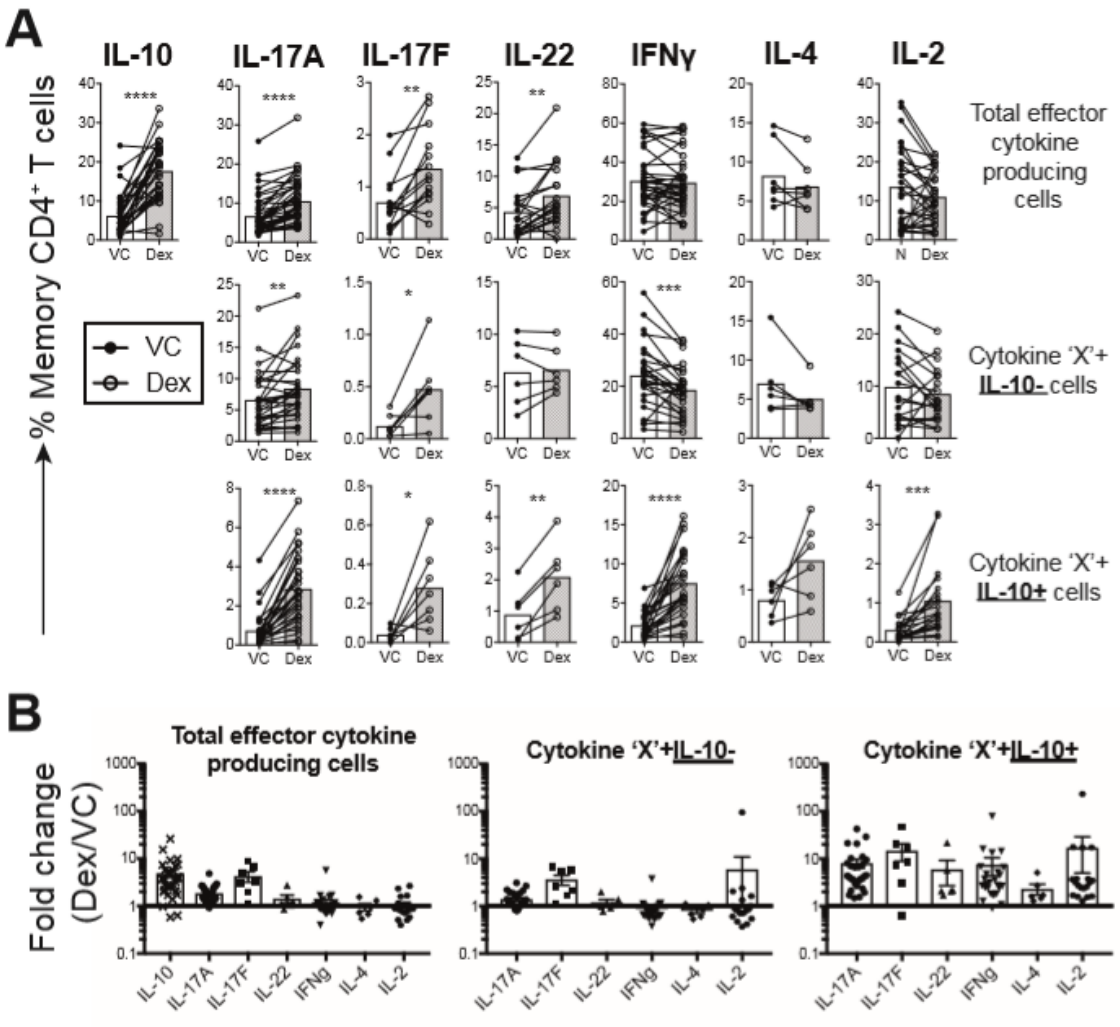


Figure 6: High dose IL-2 in the context of glucocorticoid induces maximal IL-10 production whilst inhibiting IL-17A in memory CD4$^+$ T cells

Memory CD4$^+$ T cells were stimulated with anti-CD3 alone (0 IU/ml rhIL-2), plus 10 mg/ml neutralising anti-IL-2 (aIL-2) or in the presence of the indicated concentration of rhIL-2 (50, 500 or 1000 IU/ml) in the presence of vehicle control (white bars) or 10^{-7}M dexamethasone (grey bars). On day 5 cells were stimulated for 4 hours with PMA and Ionomycin prior to performing intracellular cytokine staining. **A**, the percentage of cells expressing the indicated cytokine(s). **B**, representative plots showing expression of IL-10 relative to cell proliferation over the IL-2 titration **C**, the percentage of IL-10$^+$ cells when gating on the divided (top) or undivided (bottom) cells. n=6; ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between control and dexamethasone; # $p \leq 0.05$ and ## $p \leq 0.01$ between different IL-2 doses.



Supplementary Figure 1: Glucocorticoids enrich the proportion of IL-10+ within the memory CD4+ T cells compartment. PBMCs were stimulated with 10-7M dexamethasone or vehicle control for 5 days. Cells were then stimulated for 4 hours with PMA and Ionomycin prior to staining for surface markers and intracellular IL-10. The percentage of the indicated sub-population of cells expressing IL-10 was determined based on expression of CD4 and CD45RO (A - gating strategy). Shown are representative plots (B) and cumulative data showing the fold change in percentage IL-10+ cells with dexamethasone treatment is normalised to the vehicle control (C; n=4).



Supplementary Figure 2: Glucocorticoid specifically enrich the proportion of cells co-producing IL-10 and multiple effector cytokines.
Memory CD4+ T cells were stimulated in the presence of vehicle control or 10-7M dexamethasone. On day 5 cells were stimulated for 4 hours with PMA and Ionomycin prior to performing intracellular cytokine staining. A, the percentage of memory CD4+ T cells producing the indicated cytokine, presented as total (top), IL-10- (middle) and IL-10+ (bottom) for each donor (IL-10 n=32; IL-17A n=29; IL-17F n=7; IL-22 n=6; IFN⊠ n=25; IL-4 n=6; IL-2 n=19). Data assessed by paired-t-test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. B, fold change in the frequency of cytokine producing cells in dexamethasone-treated cultures as compared to the vehicle control for total (left), IL-10- (middle) and IL-10+ (right) cells.